Progression of staining-type hypermelanosis on the blind side in normally metamorphosed juveniles and pigmentation progression in pseudoalbino juveniles of the Japanese flounder Paralichthys olivaceus using individual identification

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Abstract Abnormal flounder coloration frequently occurs in flounder hatcheries and diminishes the commercial value of the fish. To understand hypermelanosis, the progression of staining-type hypermelanosis in normally metamorphosis juveniles and ocular-side pigmentation in pseudoalbino juveniles were examined in the Japanese flounder *Paralichthys olivaceus.* Sixty-five days post hatching, juveniles (total length, 6 cm) were individually identified by color-marker implantation, and the darkened area of the body surface was examined for 10 weeks by image analysis of digital photographs of the fish taken from the above or below the transparent tank. Staining was observed to mainly begin at the upper and lower bases of the tail fin, expand anteriorly along the peripheral part of trunk, and ceased after 2 months. The individuals in which staining occurred earlier expressed severe staining and small body size by the end of experiment. Further, pigmentation of the ocular side in pseudoalbino juveniles ceased after 2 months, but the order of pigmentation was different from that on the blind side. In this case,

31 darkening began from the posterior, but expanded from the center to the periphery of the trunk. Even

32 at the end of experiment, ctenoid scales were exclusively found within the darkened area, together with

33 cycloid scales.

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35

36 **Keywords** color anomaly, ctenoid scale, hatchery production, hypermelanosis, individual identification,

37 Japanese flounder, pseudoalbino, staining.

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39 Introduction

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41 The production of juvenile Japanese flounders *Paralichthys olivaceus* is successful on an industrial scale

42 in hatcheries [1]. However, the occurrence of “staining” remains an uncontrolled problem. Staining is

43 a type of color anomaly and is expressed as darkened areas on the blind side of fish after the completion

44 of metamorphosis [2], which decreases the market price of the fish [3]. Previous studies on staining and

45 related phenomena have shown that bottom sand is a preventive measure for staining [4-6]. In addition,

46 information which suggests the morphological similarity between staining and normal ocular side have

47 been accumulated. For example, in the stained area of blind side, there are adult-type melanophores,

48 xanthophores, and ctenoid scales; those normally appear only on the ocular side during or after

49 metamorphosis [7-14]. For "true ambicoloration", different type of darkening that visible even just after

the completion of metamorphosis, Seikai had proposed the idea that the darkening of this type is due to ocular side formation on the blind side [15]. In our previous study confirming the type and location of chromatophores and scale types (cycloid and ctenoid), we proposed the fundamental nature of staining as a “status change in the body surface conditions from the blind to the ocular side” [14].

However, the detailed process and duration of staining expansion is still unclear. Furthermore, although presence and local expansion of ctenoid-scale area had been partially reported inside of the stained area [10, 14], quantitative analysis of ctenoid-scale area, cover ratio of ctenoid in stained area for example, has not been accomplished yet. They are mainly because of the large individual variance in the extent of staining; the area of staining can vary from 0% to 50% of the blind side of fish in a single rearing tank [14]. In the time-course samplings employed in most of the previous studies [4-6, 9-14], including ours, individual organisms were killed at each time point; thus, at each time point a different

fish was assessed. Therefore, it was methodologically impossible to precisely reconstruct the actual staining process that occurred in an individual. By using a large sample (60 individuals per sampling date), although Kang and Kim first described the change in stained-area ratio with time on the blind side of a fish, which is a quantitatively precise indicator of the degree of staining [13], their final sampling was conducted at 3 months post hatching, when the staining area was still increasing. Hence, the duration of staining progression has not been examined precisely yet enough to discuss the mechanisms of staining progression. To overcome the large individual variance in staining and determine the duration of staining progression, we used individual identification in this study following Yamada et al. [16], and traced the staining expansion process without killing the target fish. Furthermore, by observing the process more frequently than Yamada et al. [16] and by adding detailed analyses between the stained area and various possible parameters, this method allowed us to retrace the characteristics of individuals that

expressed severe staining at the end of experiment.

Pseudoalbinism is another type of color anomaly, in which fish lack pigmentation on the ocular side, and this has been largely overcome by improvements in nutrition [15, 17, 18]. In pseudoalbino juveniles, it is known that pigmentation occurs after metamorphosis on the non-pigmented area on the blind side, and morphological similarity between the pigmented area and the normal ocular side has been indicated [8, 9]. This phenomenon is very similar to the staining observed on the blind side of normally metamorphosed juveniles. Moreover, the blind side of pseudoalbino juveniles tended to show severe staining [4, 8]. Therefore, we additionally examined pseudoalbino juveniles, focusing on the expansion process of the darkened area on the ocular side as well as on the blind side, by using our individual identification method.

Materials and methods

Samples

Rearing before experiments

Fertilized eggs of the Japanese flounder were obtained by natural spawning from mature adults maintained at Chiba Prefectural Farming Center, Chiba, Japan. After hatching, larvae were reared under natural water temperature, initially at 18°C at hatching, 23°C at the completion of metamorphosis [34 days post hatching (DPH)], and 27°C just before the transportation to Kyoto University (62DPH). The rearing density was 20000 individuals /m^3 at hatching, 8000-9000 individuals /m^3 at 10 DPH, 5000

94 individual /m^3 when metamorphosis completed, and 500 - 1000 individuals / m^3 for settled juveniles.

95 Average growth rate of the juveniles was 1.3 mm / day for the period of 55 - 60 DPH. At 63 DPH, 65

96 flounders without darkened areas and 29 pseudoalbino flounders, with total lengths of approximately 6

97 cm, were selected, and transported to Kyoto University through parcel delivery service packed with

98 oxygen at natural temperature. They arrived at 64 DPH.

99

100 *Selection of individuals for experiments*

101

102 Faint staining occurred on the blind side of some individuals during transportation. To examine the

103 possible differences between no-stained and faintly-stained individuals, normally-metamorphosed

104 individuals were divided into two groups after arrival at the Kyoto University: (1) clean-started,

105 individuals with normal ocular side development and no darkened areas on the trunk of the blind side at
106 the beginning of the experiment (ratio of darkening [stated below in “Measurement of changes in
107 darkened areas”]: 0.006 - 0.014, 13 individuals); (2) stain-started, individuals with normal ocular side
108 development and darkened areas on the tail base (level 3 in Isojima et al. 2013) of the blind side at the
109 beginning of the experiment (ratio of darkening: 0.012 - 0.077, 17 individuals). In addition, individuals
110 with pseudoalbinism on the ocular side at the beginning of the experiment were classified as (3)
111 pseudoalbino (ratio of darkening on the blind side: 0.015 - 0.035, 10 individuals). For each group,
112 typical appearance on the blind side at the beginning of experiment was shown in Fig. 1. In the
113 pseudoalbino group, 7 individuals showed darkened areas on the blind side at the beginning of the
114 experiment. The remaining individuals that were not included in the above groups and not used for this
115 experiment were anesthetized in 0.1% 2-phenoxyethanol (Nacalai Tesque Inc., Kyoto, Japan) and fixed in

10% neutralized formalin (Nacalai Tesque Inc.) as the initial samples.

At 65 DPH, the juveniles for the rearing experiment were marked with 3 colors (red, blue, and green) by using visible implant elastomer (Northwest Marine Technology, Inc., Shaw Island, WA, USA) at 4 points on the blind side to enable these individuals to be identified.

Rearing procedure and final sampling

All juvenile flounders of three groups (clean-started, stain-started, and pseudoalbino) were mixed after marking, and reared together in a 100-l polycarbonate tank (transparent, installed on a gray plastic plate) with a water temperature of approximately 25 °C and were fed 4 times a day with artificial diets (Nagisa K1 [diameter, about 0.8–1.2 mm; 65–85 DPH] and Nagisa K2 [diameter, about 1.2–2.8 mm; 86–130

DPH], Oriental Yeast Co. Ltd., Tokyo, Japan; Otohime EP2 [diameter, about 1.9–2.3 mm; 131–142 DPH], Marubeni Nissin Feed, Tokyo, Japan).

Since previous studies have shown that the stained area is larger in flounders reared in tanks without bottom sand [4-6] and with longer periods of light irradiation per day [6], we reared flounders in tanks without bottom sand, with 16 h light irradiation per day. However, the density of juveniles in the tank was not adjusted in response to their growth or decrease in numbers because staining occurs at a significant level in the tanks without bottom sand regardless of density [19].

At 143 DPH, all juveniles (except 8 individuals that were used for a different experiment) were anaesthetized using 0.1% 2-phenoxyethanol (Nacalai Tesque Inc.), fixed in 10% neutralized formalin (Nacalai Tesque Inc.), and the distribution of ctenoid scales was examined.

Samples reared at Fukui Prefectural University

To examine the distribution of ctenoid scales on the blind side of juveniles after an extended period, flounders more than a year old were also examined. The flounders were originally purchased from Marinetech Co., Ltd. (Tahara, Japan) as juveniles and were reared in tanks without bottom sand at Fukui Prefectural University Research Center for Marine Bioresource, in accordance with the ordinary flounder-rearing procedure used in this laboratory. Six flounders of age about 1 year were randomly selected as samples, anesthetized using 0.1% 2-phenoxyethanol (Nacalai Tesque Inc.), and fixed in 10% neutralized formalin (Nacalai Tesque Inc.).

Measurement of changes in darkened areas

149

150 The blind side of each flounder without anesthetizing was photographed once a week from 66 to 136

151 DPH by using a digital camera (OLYMPUS Tough TG-615, Olympus Corp., Tokyo, Japan) installed

152 under the transparent glass aquarium. The size of the darkened areas, the size of the blind side

153 (excluding the fins), and body length were measured using NIH Image J (available online at

154 http://rsbweb.nih.gov/ij/; National Institute of Health, USA). For pseudoalbino fish, the size of the

155 darkened area on the ocular side and the size of the ocular side (excluding the fins and eyes) were

156 measured after the first week. By using these results, the ratio of darkening on the blind side of each

157 individual (size of the darkened area on the blind side/size of the blind side, excluding the fins) and the

158 ratio of darkening on the ocular side of each individual (size of the darkened area on the ocular side/size

159 of the ocular side, excluding the fins and eyes) were calculated. The measurements were terminated at

136 DPH (10th week) because the staining expansion appeared to cease in all individuals, and the ratio of darkening on the blind side at 136 DPH was regarded as the maximum ratio of darkening. Daily growth rate (mm/day) was calculated for the experimental period of 10 weeks.

Because an obvious increase in darkened area was observed during the period when the ratio of darkening on the blind side ranged from 10% to 90% of the maximum ratio of darkening, this period was considered as the darkening period. Further, the increase in the ratio of darkening with time (per weeks) during this period was calculated as the darkening speed as follow.

$$\frac{(\text{ratio of darkening at the end}) - (\text{ratio of darkening at the begining})}{(\text{weeks between the beginning and end of the period})}$$

Examination of the distribution of ctenoid scales

171 To clarify the relationship between staining and shift from cycloid scales to ctenoid scales on the blind
172 side of juvenile flounder, the distribution of ctenoid scales was extensively examined across the entire
173 blind side by using the method described by Isojima et al. [14].

174 From the stain-started juveniles reared at the Kyoto University, 6 individuals at 143 DPH with a
175 typical degree of staining for the group at that age (ratio of darkening: 0.42-0.50, appearance similar to
176 the illustrations of 4th - 5th week in Fig. 3) were selected for this examination and were termed as
177 “having finished staining.” In addition, all flounders reared at Fukui Prefectural University were used in
178 this examination and were named “1-year-old” (ratio of darkening: 0.29-0.93).

179 Following the method of Isojima et al. [14], spines of ctenoid scales were visualized. In brief,
180 formalin-fixed whole body samples were stained with Alizarin Red, immersed in 70% ethanol, air dried.
181 Then, the spines of ctenoid scales reflect light irradiated from anterior direction. On photographs taken

for whole blind side, the area of the blind side (excluding the fins), darkened areas, darkened areas occupied by ctenoid scales, and normal areas occupied by ctenoid scales were measured from the digital images using NIH Image J. By using these results, the ratio of darkening and the ratio of ctenoid scales in the darkened area (the ratio of areas occupied by the ctenoid scales in the darkened area) were calculated.

Statistical analysis

For statistical analyses, online tools provided by the Osaka University (available at http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom) were used. Student's *t* tests followed by subsequent multiple comparisons using the Tukey–Kramer method were used to compare growth, daily

193 growth rate, maximum ratio of darkening, and darkening speed among each group.

194

195 Results

196

197 Comparison of growth among the three groups

198

199 The increase in body length per day of the flounders reared at Kyoto University ranged from 0.7 to 1.4

200 mm/day, and the mean was 1.1 ± 0.03 mm/day. Although we attempted to select individuals of

201 uniformed body length before transporting the fish to Kyoto University, pseudoalbino fish were

202 significantly larger than stain-started fish at the beginning of the examination ($P < 0.05$, Table 1). At the

203 end of the measurements (10^{th} week), however, clean-started fish were significantly larger than the other

204 fish. This was same about growth per day ($P < 0.05$; Table 1).

205 At the individual level, no obvious relationships were observed between the body length and

206 maximum ratio of darkening for individuals of both clean-started and stain-started groups ($R^2 = 0.26$, Fig.

207 2).

208

209 Qualitative and quantitative analysis of darkening enlargement on the blind side

210

211 Figure 3 shows the typical enlargement of staining on the blind side of a stain-started individual. In all

212 the individuals, staining began from the base of tail fin, extended anteriorly along the base of the dorsal

213 and anal fins, and extended from the edge to the lateral line. Furthermore, in some individuals, after the

214 darkening reached around the middle of the trunk, it began at three portions, including the bases of the

pectoral fin, pelvic fin, and the center of the head. The former two spots expanded until 2 areas were connected, and the spot in the head expanded concentrically. It was noticed that all the individuals at the end of experiment showed a similar pattern of darkened area to either one of the figures shown in Figure 3.

Regardless of the groups, the ratio of darkening on the blind side increased with time up to a certain value (Fig. 4). No obvious relationship was observed between the ratio of darkening at the start of the experiment and maximum ratio of darkening (Table 2). However, in the measurements after the 5^{th} week, the ratio of darkening showed a strong linear relationship with the maximum ratio of darkening and the contribution ratio of regression (R^2) was over 0.9 (Table 2).

However, as shown in Figure 4, the increase in the ratio of darkening in each individual had ceased before the final measurement, about 1–2 months after the beginning of the darkening period. The

226 darkening period of clean-started fish tended to be longer and ceased later than that in the fish of the other
227 groups.

228 The maximum ratio of darkening largely differed, even among individuals in the same group.
229 However, when comparing the groups, the maximum ratio of darkening of clean-started fish (0.21 ± 0.04)
230 was significantly smaller than that of the stain-started (0.42 ± 0.03) or pseudoalbino fish (0.48 ± 0.05) (P
231 < 0.05; Fig. 5a). A similar trend was observed for the darkening speed (P < 0.05; Fig. 5b). For
232 individuals that had metamorphosed normally (clean-started and stain-started), a strong linear relationship
233 was observed between the maximum ratio of darkening and the darkening speed (Fig. 6).

234

235 Darkening on the ocular side of pseudoalbino fish

236

There were 2 types in the order of darkened area on the ocular side darkening of pseudoalbino fish (Fig. 7). In 6 individuals (type A), the darkening of the head occurred after complete darkening of the trunk. In the other 4 individuals (type B), the darkening of the head occurred simultaneously with the trunk darkening. In both the types, darkening hardly occurred at the anterior base of the dorsal fin. During the course of darkening, the darkened area on the trunk expanded from the tail to the head in a typical manner, as observed during the course of darkening on the blind side of the fish. However, in contrast, the darkening on the ocular side of pseudoalbino fish vertically expanded from the lateral line to the edge (Fig. 7). In addition, it was characteristic to ocular side darkening that pale darkening occurred initially, and then the area gradually became darker and finally reached a similar coloration to the normal ocular side (data not shown).

The ratio of darkening on the ocular side of pseudoalbino fish increased with time (Fig. 8).

248 Within 10 weeks, the increase in the darkened area stopped with the ratio of darkening greater than 0.9.

249 There were no obvious differences in the ratio of darkening or darkening speed between type A and type

250 B individuals (Fig. 8).

251

252 Distribution of ctenoid scales on the blind side

253

254 On the blind side of all examined individuals, the presence of ctenoid-scale-covered areas was confirmed

255 almost exclusively within darkened areas, together with cycloid-scale-covered areas, as observed on the

256 normal blind side. The ratio of ctenoid scales in the darkened area varied from 10% to 70%, even

257 among individuals in a tank (Fig. 9). Examining the relationship between the ratios of ctenoid and

258 darkening, a strong linear relationship was observed, but only among individuals in the same rearing (Fig.

259 9).

260 For the location of the ctenoid-scale area at the end of experiment, similarity to the darkened

261 areas at a certain time point in the past was observed (Fig. 10 shows a typical example). The

262 ctenoid-scale area at the 11th week (shaded area in the upper panel) resembles the stained area at the 3rd

263 week (black area in the lower panel). Such similarities appeared stronger in individuals having smaller

264 darkened areas. The time point in the past at which the strongest similarity was observed was different

265 according to individuals, but was within the range of the 3rd to the 6th week.

266

267 Discussion

268

269 Changes in location and ratio of darkening on the blind side

From the results, almost all darkening on the blind side of juveniles in the present study occurred after the completion of metamorphosis and the beginning of the experiment, as shown in Figure 4. Therefore, we have used “staining” for the darkening phenomenon on the blind side in the discussion section. Because the ratio of darkening increased gradually in all individuals and staining tended to appear next to existing stained areas, it is obvious that the stained area expanded gradually to the neighboring areas. In addition, as Figure 3 indicates, the order of appearance was as follows: (1) starting from the tail base and expanding anteriorly; (2) starting from the base of pectoral and pelvic fins and expanding until 2 areas were connected ; and (3) starting at the center of the head and expanding concentrically. So, although such a pattern of expansion was expected using observations from the time-course sampling [13, 14] and classifications of staining extent [19, 20], to our knowledge, this is the first report directly confirming the

281 detail time-course of the expansion process in detail using individual identification.

282 Although, Kang and Kim showed the increase in individual staining ratio with respect to time

283 [13], the complete duration of staining progression was unclear. In the present study, the increase in the

284 ratio of darkening decreased and ceased at about 8^{th}–10^{th} weeks in all individuals, and the ratio of

285 darkening extensively differed among individuals, as shown in Figure 4. From this result, it is obvious

286 that the progression of staining does not continue until adulthood, but only continues for about 2 months.

287 This possibility was previously suggested [6, 14] and is further confirmed by the present study.

288 There are 2 possibilities for the stasis of staining expansion. One possibility is time

289 limitation; the staining cannot progress longer than 2 months after the first appearance or 20 weeks after

290 hatching. The second is area limitation; the maximum area of staining is individually prefixed, and no

291 further progression of staining occurs after the individually prefixed maximum area is reached.

292 Although we cannot conclude from the results of the present study, we consider that the latter is more

293 probable. As Figure 6 indicates, the maximum ratio of darkening was almost proportional to the

294 darkening speed. Therefore, in the individuals that would have larger stained area, the staining speed is

295 fast from the beginning of the staining expansion. This suggests that the maximum ratio of darkening of

296 each individual had been decided before the beginning of staining, and may support the presence of

297 prefixed area of staining. .

298

299 Comparison between clean-started and stain-started groups

300

301 The maximum ratio of darkening in stain-started fish was significantly larger than that in clean-started

302 fish (Fig. 5a, $P < 0.05$). A similar result was observed for darkening speed as shown in Figure 5b.

303 Interestingly, at the end of the experiment, the body length of stain-started individuals was
304 significantly smaller than that of clean-started individuals (Table 1). Although stain-started individuals
305 had a larger maximum ratio of darkening as shown in Figure 5a, a direct relationship between stained area
306 size and body length is not observed at the individual level (Fig. 2). This result suggests that the smaller
307 final size of stain-started individuals was more closely related to the early start of staining rather than the
308 large size of the final stained area. Therefore, individuals showing a slow start to staining are not
309 expected to develop severe staining, and at the same time, are expected to grow better. Although more
310 confirmation is needed, the timing of the first appearance of staining on the blind side of juveniles may be
311 utilized as an index of individual quality; the later appearance of staining indicates a better quality
312 individual, for both the extent of staining and growth. To date, information on the causality between
313 growth and staining is limited.

314 It was previously reported by Seikai [4, 8] that pseudoalbino flounders tended to have severe
315 staining on the blind side. However, in our results, the extent of darkening on the blind side of
316 pseudoalbino juveniles was similar to that observed in the stain-started individuals. In addition, no
317 difference was observed in the process of staining on the blind side between the two groups.
318
319 Ocular side darkening of pseudoalbino individuals
320
321 In this study, more than 90% of the ocular side of juveniles showed pigmentation by the end of
322 experiment. Although darkening on the ocular surface was almost uniform and lacked black or white
323 circle patterns, the darkness and hue was almost similar to that of normal fish (data not shown). This
324 observation is in accordance with Seikai [8], who showed that the ocular side of pseudoalbino individuals

eventually becomes similar to that of normal fish. Since staining of the blind side was considered to be same as a normal darkening process of ocular side skin [14], darkening of the ocular side of pseudoalbino flounders is possibly the same phenomenon as staining on the blind side. However, the direction of vertical extension was different between the two; pigmentation extended from the lateral line to the edge of the trunk in ocular side darkening of pseudoalbino juveniles (Fig. 7), while pigmentation extended from the edge to the lateral line in blind side staining of normal juveniles, as shown in Figure 3 and as previously reported [14]. Therefore, the controlling systems of pigmentation are possibly different between staining on the blind side and darkening on the ocular side of pseudoalbino juveniles.

Ikuta [21] divided darkening of the ocular side of pseudoalbino flounders into 2 types according to the pattern of the darkened area. In the normal-color type (NC type), the darkened area formed a similar pattern to that of the normal ocular side, while in the abnormal-color type (AC type), the

darkened area was uniformly dark brown with no characteristic patterns of Japanese flounder. However, both Type A and Type B in the present study showed uniform coloration without specific patterns, suggesting that both A and B types are basically defined as AC type. Further, Ikuta [21] reported the difference in the darkeni[ng process between the two types; the head quickly darkened in the NC type, while the head was barely darkened in the AC type. For this point, Type A and Type B fish in the present study resembled the AC type and NC type, respectively. Although not mentioning to color pattern on the darkened area nor the presence of NC type, Terui [22] pointed out the similarity in darkening process to AC type in their study using mud dab *Limanda yokohamae*. In addition, although the darkening speed of NC type fish was higher than that of AC type fish [21], such a tendency was not observed in this study (Fig. 7). The biological significance as well as the presence of 2 types of darkening are not clear and require further information.

347

348 Process and regulation of ctenoid scale formation on the blind side

349

350 Normally, ctenoid scales are only present on the ocular side of flounder, but they have also been observed

351 on the darkened area of the blind side of juveniles [9-11, 14]. In addition, it was reported that ctenoid

352 scales with fewer spines were found on the stained area near the boundary to the normal area [9, 14].

353 Although a significant part of the stained area was covered with cycloid scales, very little of the normal

354 (white, not darkened) area of the blind side was covered with ctenoid scales, as previously reported [9-11,

355 14]. This is probably in accordance to Kikuchi et al. [7], who suggested that the putative factor(s) that

356 induces ctenoid scale formation is only effective on the darkened area, based on the observation of the

357 first formation of ctenoid scales on the normal ocular side.

Their idea was further supported, at least partially, from our observations. The ctenoid scale area at the end of experiment was similar to the stained area 3–6 weeks previously, as indicated in Figure 10. Since staining gradually expands to the neighboring area, as shown in Figure 3, it is suggested that the shift to ctenoid scales follows pigmentation. In addition, strong linear relationships were observed between the ratio of darkening and the ratio of ctenoid scales in the stained area within the group from the single rearing trial (Fig. 9). Therefore, it is expected that staining and a shift to ctenoid scales have a strong relationship, probably in terms of the blind side showing “a shift to the ocular side,” as previously suggested [14].

The expansion of staining stopped before the five months after hatching (10 weeks after the beginning of the experiment) under rearing without bottom sand, as shown in Figure 4. Therefore, 1-year-old fish is considered to have spent more than 7 months after the completion of staining.

Although information is lacking on the period required for the completion of ctenoid scale formation on the stained area, we consider that this period of 7 months is sufficient because this period is more than the period from hatching to staining stopping in the rearing experiment (less than 5 months, as above). However, no individuals were observed whose stained areas were completely occupied by ctenoid scales, suggesting that the shift to ctenoid scales stops independently from that of staining.

The strong linear relationship between the ratios of ctenoid scales and stained area was also reported by Isojima et al. [14], with a regression line ($y = 1.8812x + 0.0958$; $R^2 = 0.7162$) different from that in the present study, regardless of the sampling date (100 DPH and 120 DPH). Therefore, it is possible that a certain factor unique to each rearing trial determines the regression line by determining the end-point of the shift to ctenoid scales.

These considerations suggest that darkening is regulated slightly different from that of the final area

of ctenoid scale formation. Since the black coloration, not the presence of ctenoid scale, on the blind side decreases the market price, the effort to clarify the factors and prevent the occurrence of black coloration is critical for industrial purposes. However, at the same time, studies on ctenoid scale formation on the blind side contribute the fundamental understanding of ocular–blind side differentiation from scientific interests.

Possibility of staining degree prediction.

In this study, expansion of staining ceased by 136 DPH (10th week), as shown in Figure 4. In addition, by using individual identification, it was first clarified that the order of staining degree was almost fixed among individuals by 101 DPH (5th week), as indicated in Table 2. Therefore, for the “comparison” of

391 staining degree among experimental groups, it is possible to measure the stained area at approximately
392 the four months after hatching, although it is not clear from the present study whether age (DPH or days
393 post metamorphosis completion) or period after the beginning of staining is critical. For the
394 determination of the absolute degree of staining, measurements at 5 months after hatching seemed
395 sufficiently long. We consider this information of good use for designing an experiment on staining.

396 The first appearance of staining has been proposed as a possible indicator of future staining degree
397 in each individual because the maximum ratio of staining was significantly greater in the group where
398 staining appeared earlier (Fig. 5a). In addition, examining the first appearance of staining in individual
399 fish was not difficult. Thus, it might be possible to identify and exclude bad-quality juveniles (severe
400 staining and slow growth in the future) by early and significant appearance of staining on the blind side.
401

402

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404

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408

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491 Figure captions

492

493 Fig. 1 Typical appearance of the blind side at the beginning of experiment. a) clean-started (51 mm,

494 body length), b) stain-started (49 mm), and c) pseudoalbino (57 mm). . White bars indicate 1 cm.

495

496 **Fig. 2** Relationship between maximum ratio of darkening at the blind side and body length at the 10th

497 week for individuals that had undergone normal metamorphosis. Open circles and closed circles

498 indicate clean-started and stain-started individuals, respectively

499

500 **Fig. 3**Typical pattern of staining expansion. Black area indicates the darkened areas. The ratio of

501 darkening at the blind side is indicated next to the weeks. Stain-started individual, 5.4-cm body length

502 at 0 week and 12.4-cm body length at 10th week

503

504 **Fig. 4**Changes in the individual ratio of darkening at the blind side and the darkening period. Open

505 squares and open circles indicate the beginning and the end of the darkening period, respectively.

506

507 **Fig. 5** Comparison of (a) maximum ratio of darkening and (b) darkening speed (increase in the ratio of

508 darkening per week) among groups. Mean ± standard error (SE). Different characters indicate the

509 presence of statistical difference ($P < 0.05$)

510

511 **Fig. 6** Relationship between maximum ratio of darkening and darkening speed (increase in the ratio of

512 darkening per week) for individuals that had undergone normal metamorphosis. Open circles and closed

circles indicate clean-started and stain-started individuals, respectively

Fig. 7 Typical 2 patterns of darkening expansion on the ocular side of pseudoalbino individuals. Black areas indicate darkened areas. Head darkened after trunk in Type A, while simultaneously with trunk in Type B

Fig. 8 Change in the individual ratio of darkening on the ocular side of pseudoalbino fish. Open squares and closed squares indicate “Type A” and “Type B” individuals, respectively

522 **Fig. 9** Relationship between the ratio of darkening and the ratio of ctenoid scales in the darkened area.

523 Closed circles indicate “having finished staining” individuals (n = 6), and closed triangles indicate

524 “1-year-old” individuals (n = 6)

525

526 **Fig. 10** Typical example showing the similarity between the ctenoid-scale area at the 11th week (a) and

527 darkened area in the past (b; 3rd week). Black area indicates darkened area and slashed area indicates

528 ctenoid-scale area at the 11th week

529

530 Table captions

531

532 **Table 1** Comparison of the initial, final body lengths (cm) and daily growth rate (mm/day) among the

533 three groups

534 (foot note) *Mean ± standard error (SE) (n). Different characters indicate the presence of a statistical

535 difference among groups in each week ($P < 0.05$)

536

537 **Table 2** Regression line and R^2 values of each week between ratio of darkening (x) and maximum ratio of

538 darkening (y) of clean-started and stain-started fish

Fig. 1

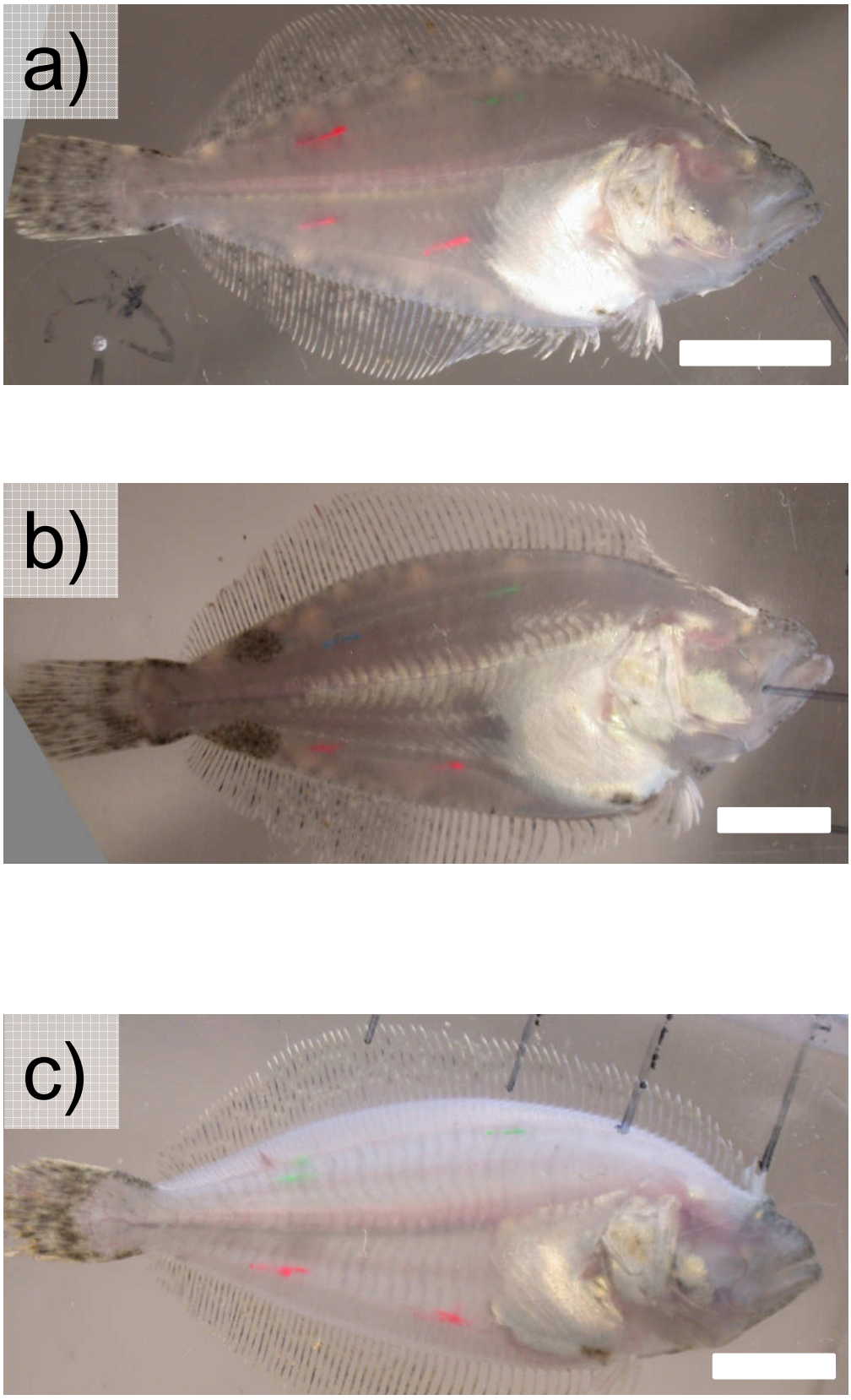
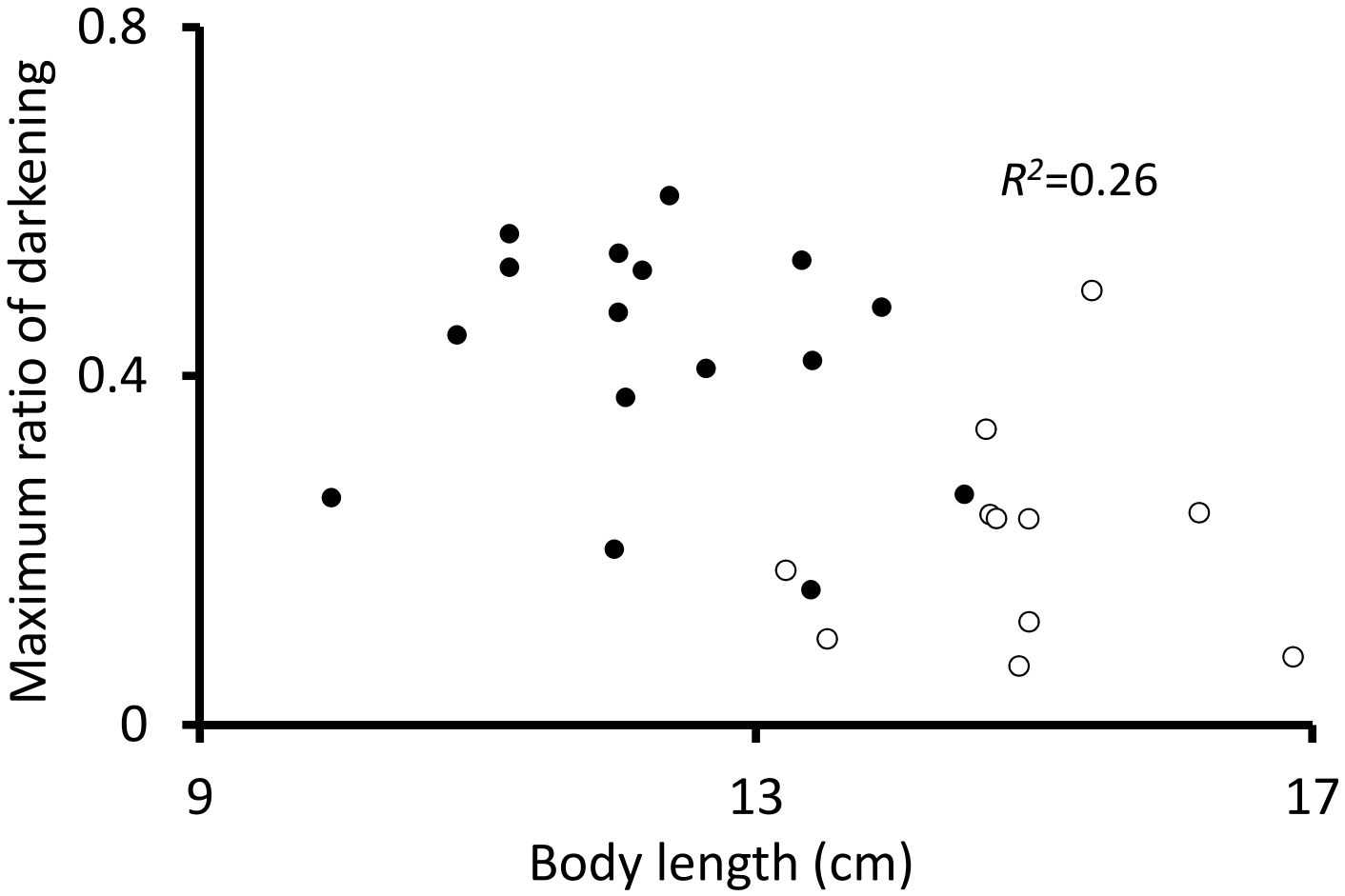


Fig. 2



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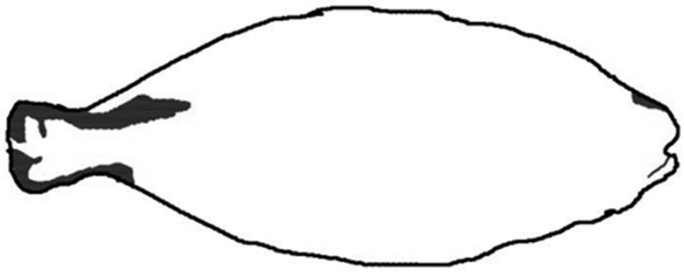
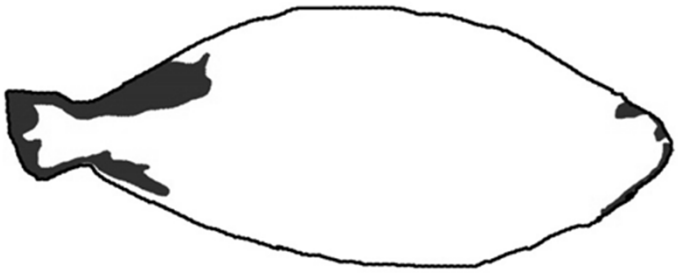


0week, 0.031

6th week, 0.544



1st week, 0.066

7th week, 0.563

2nd week, 0.205

8th week, 0.585

3rd week, 0.29

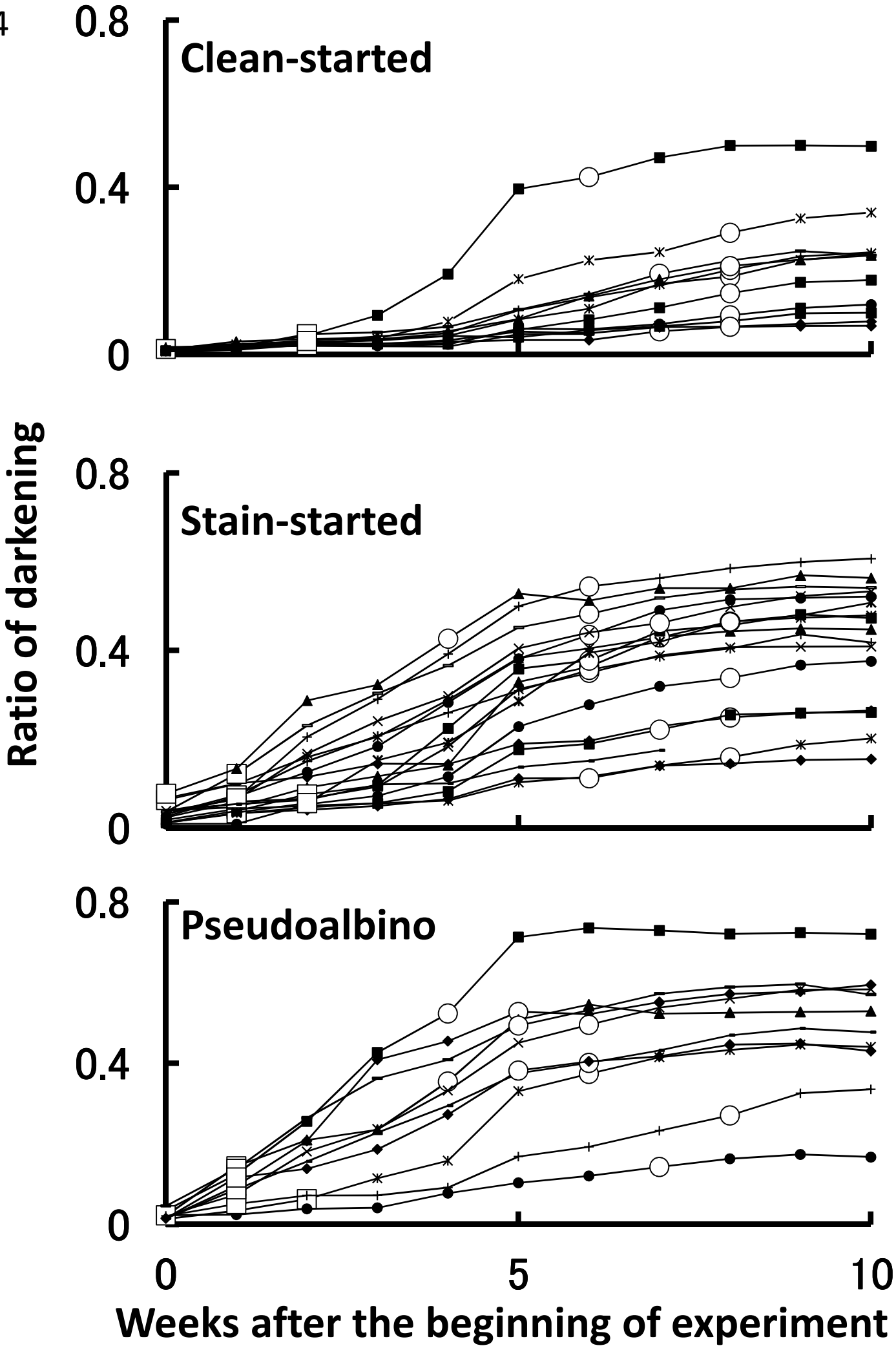
9th week, 0.599

4th week, 0.392

10th week, 0.607

5th week, 0.5

Fig. 4



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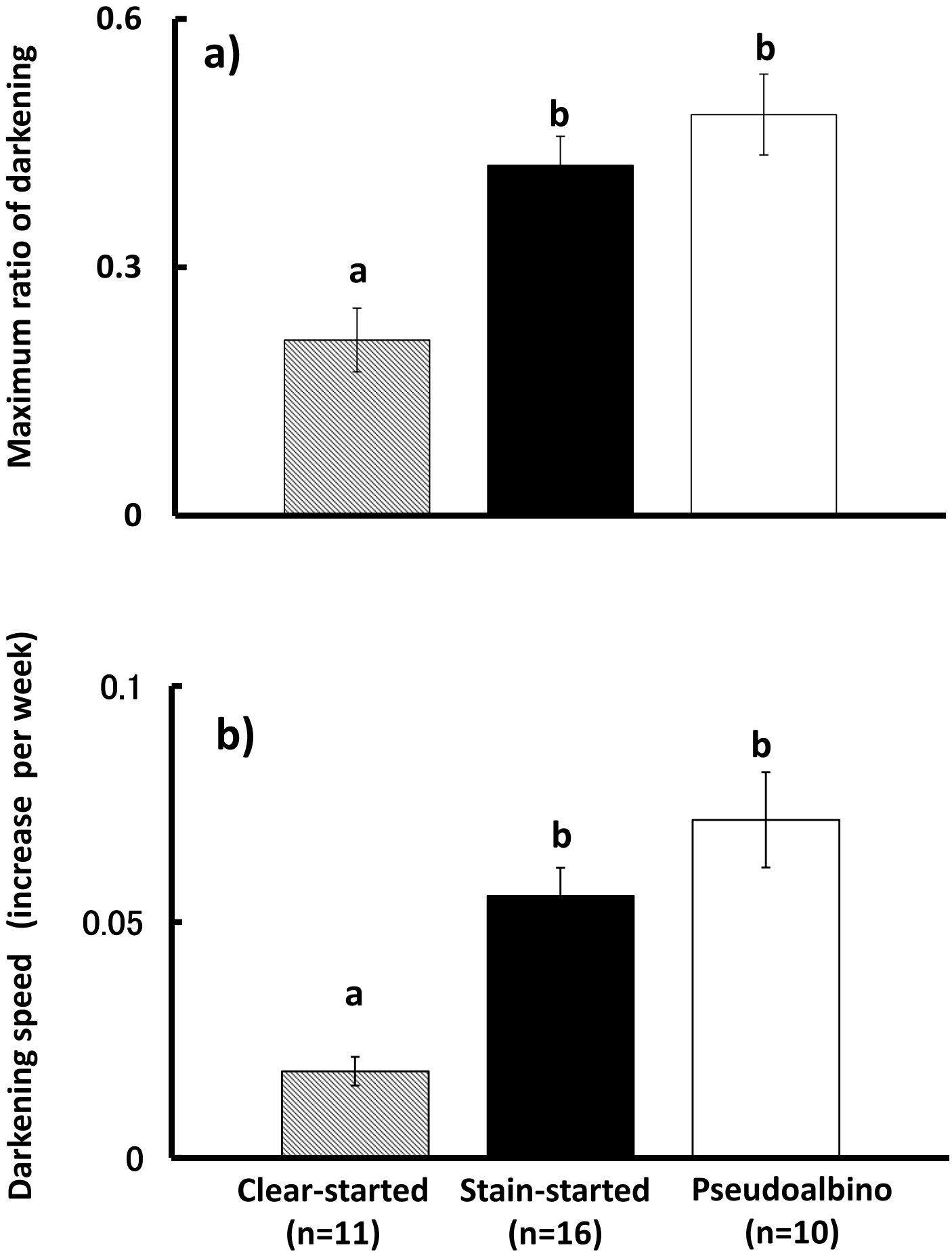


Fig. 6

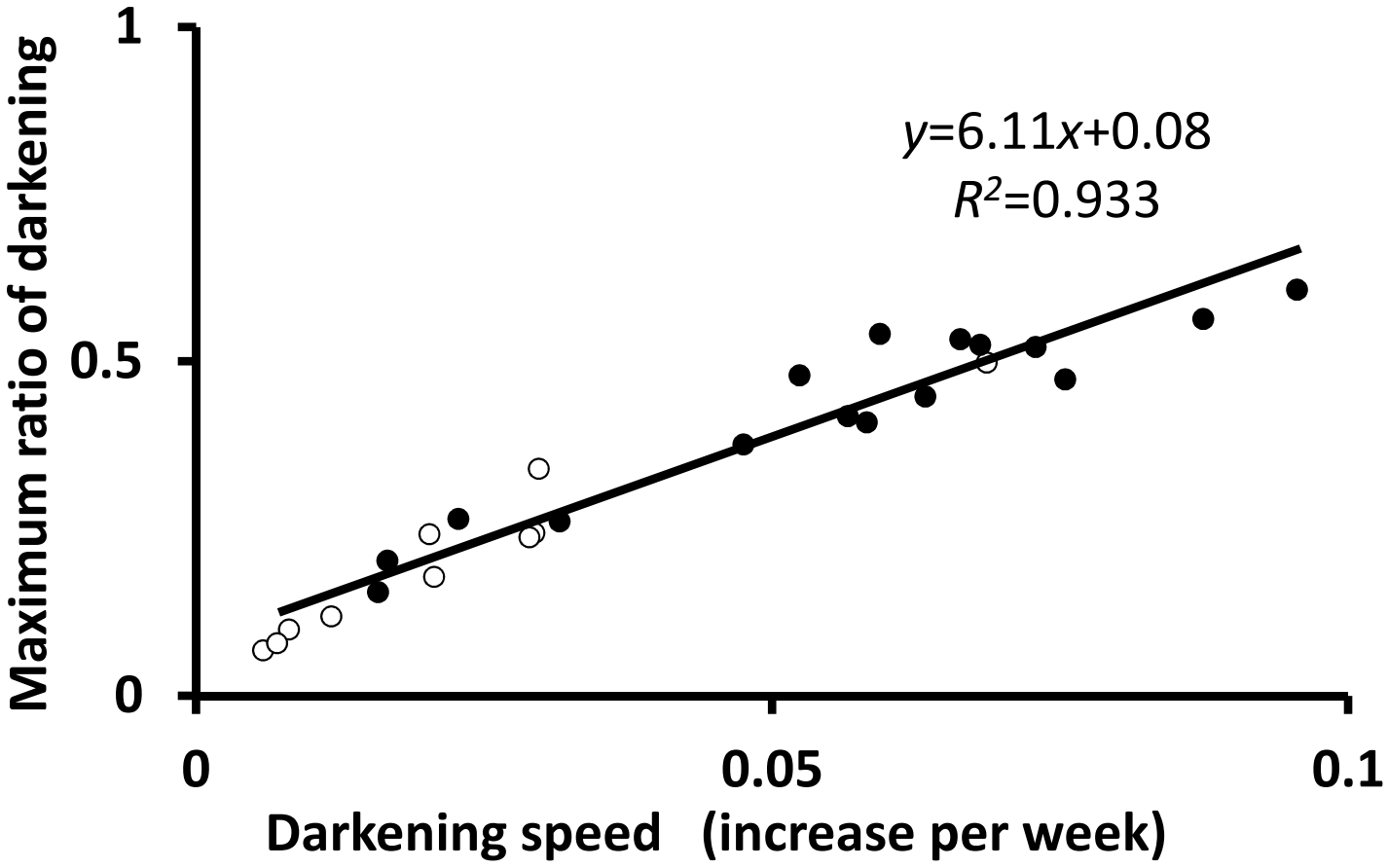


Fig. 7

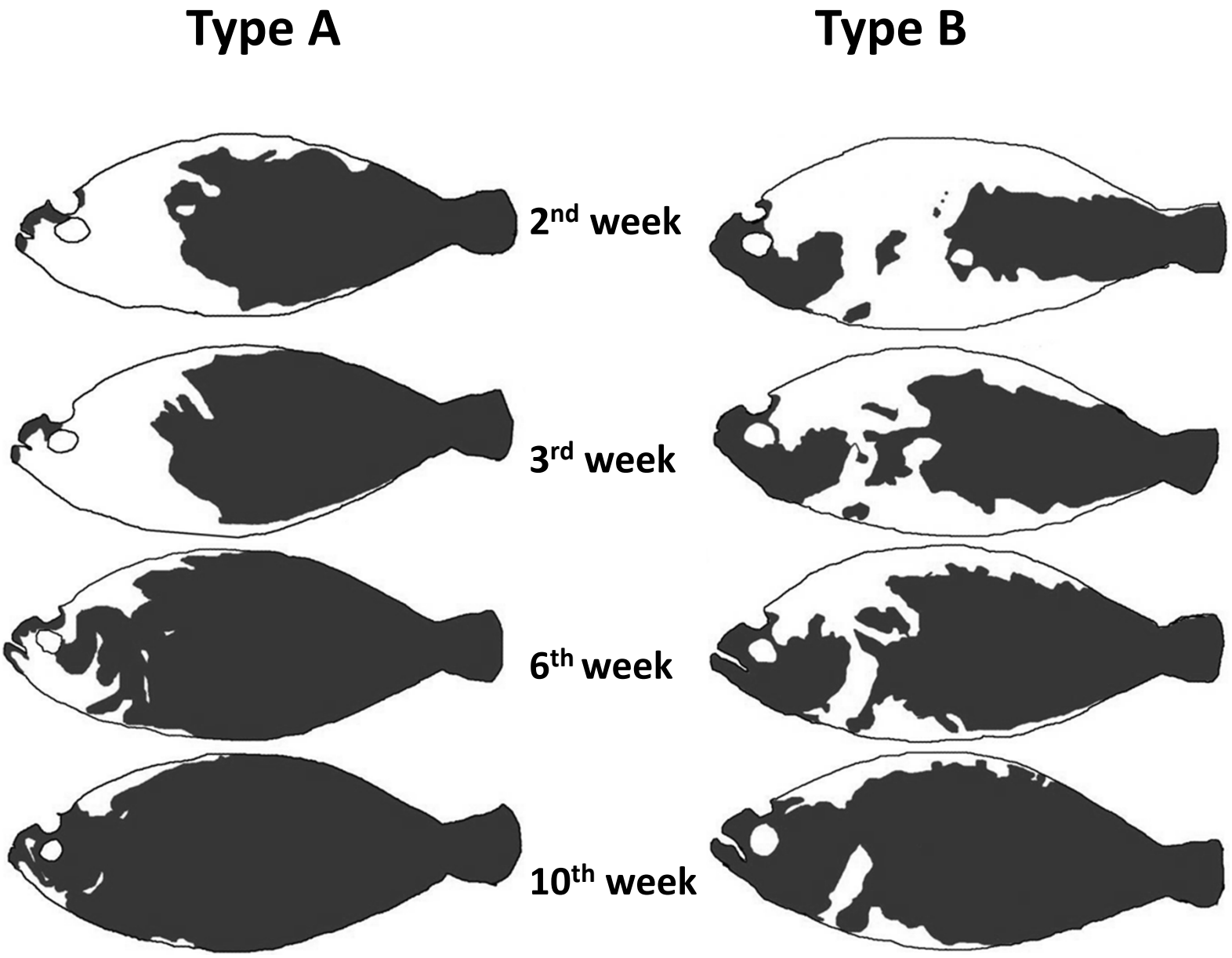


Fig. 8

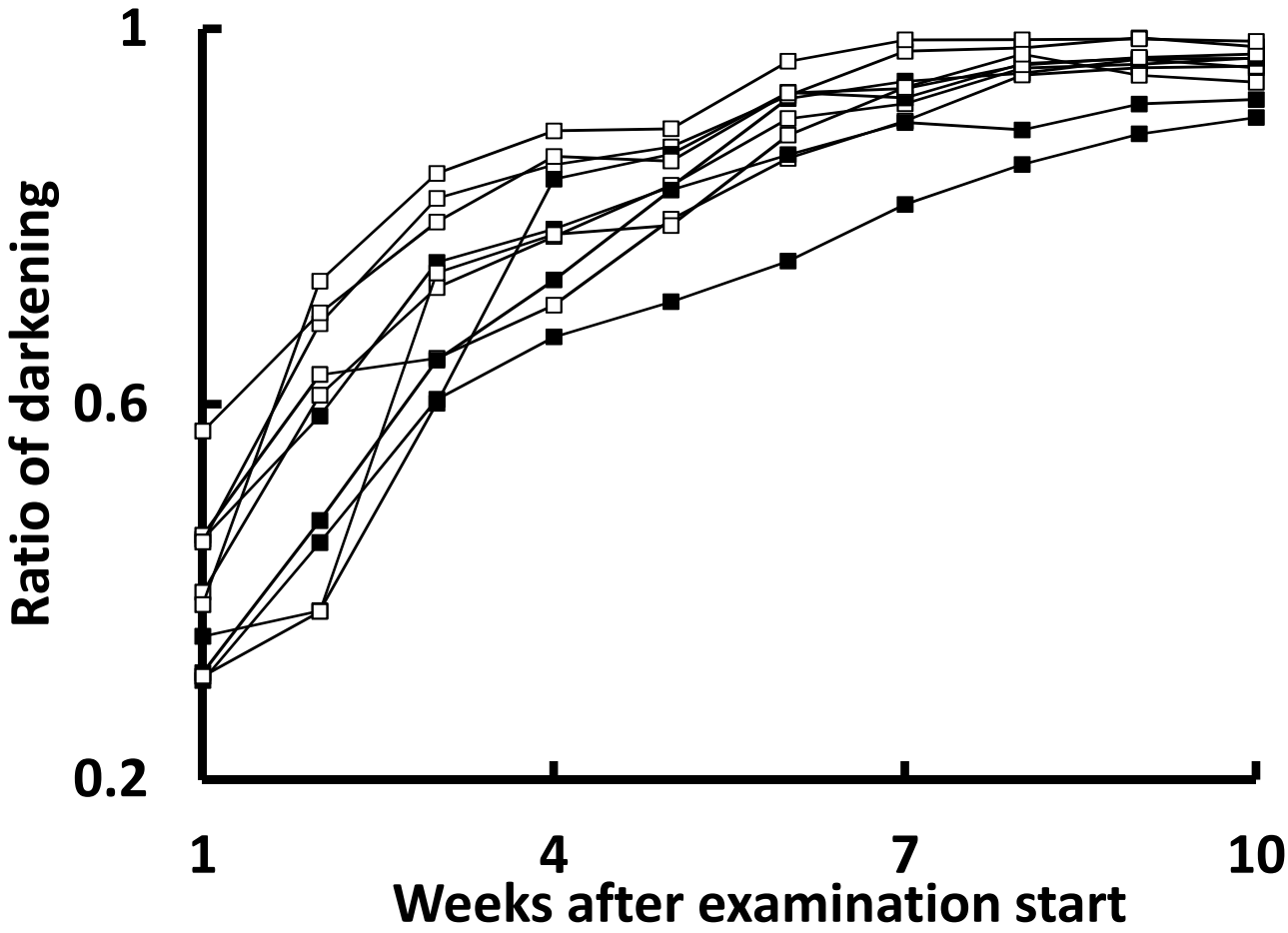


Fig. 9

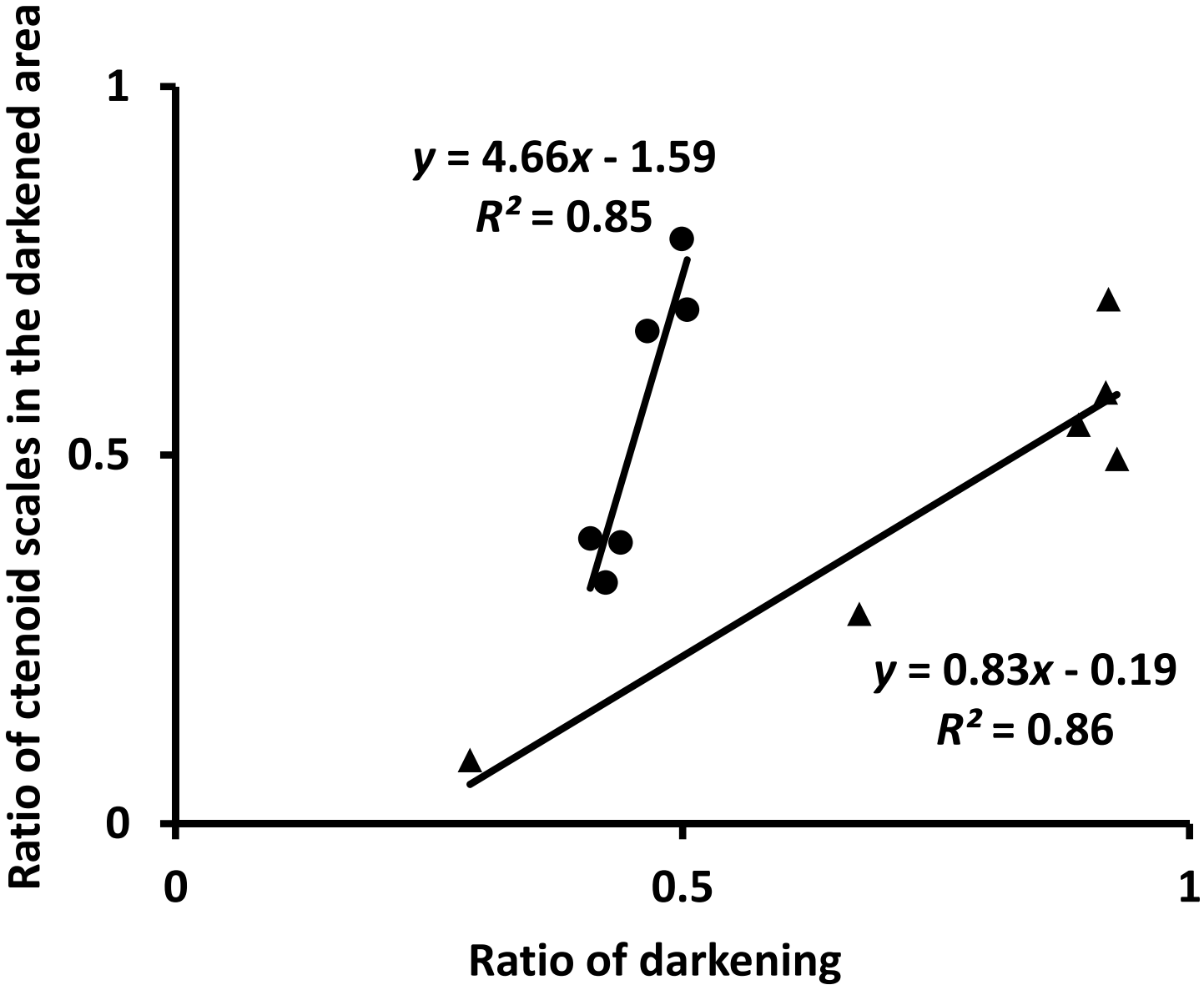
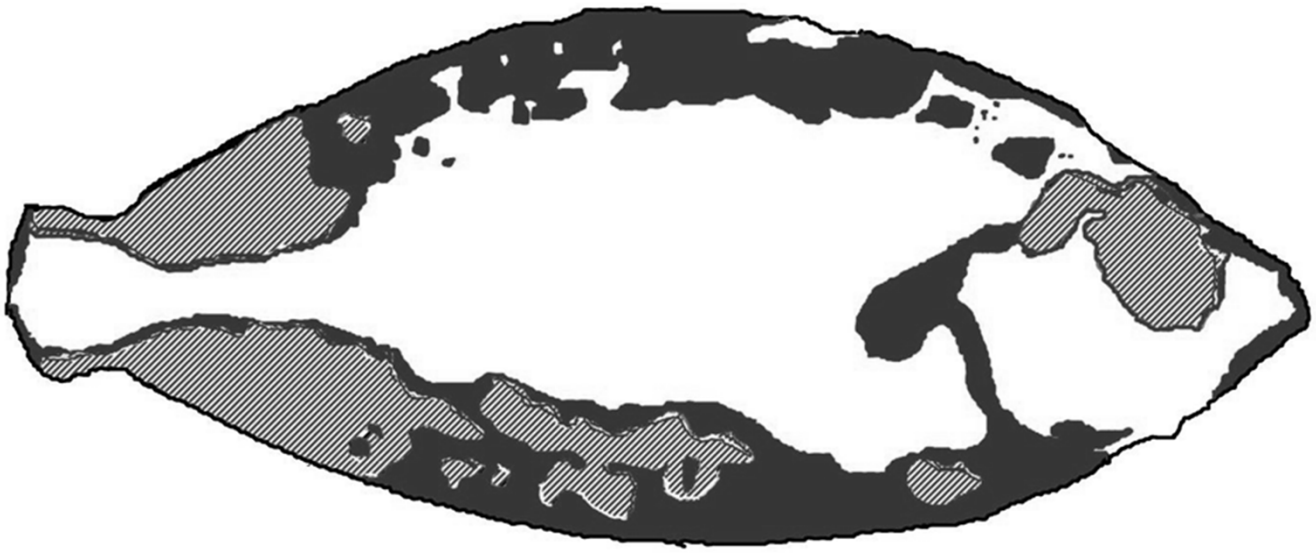


Fig. 10

a)



b)

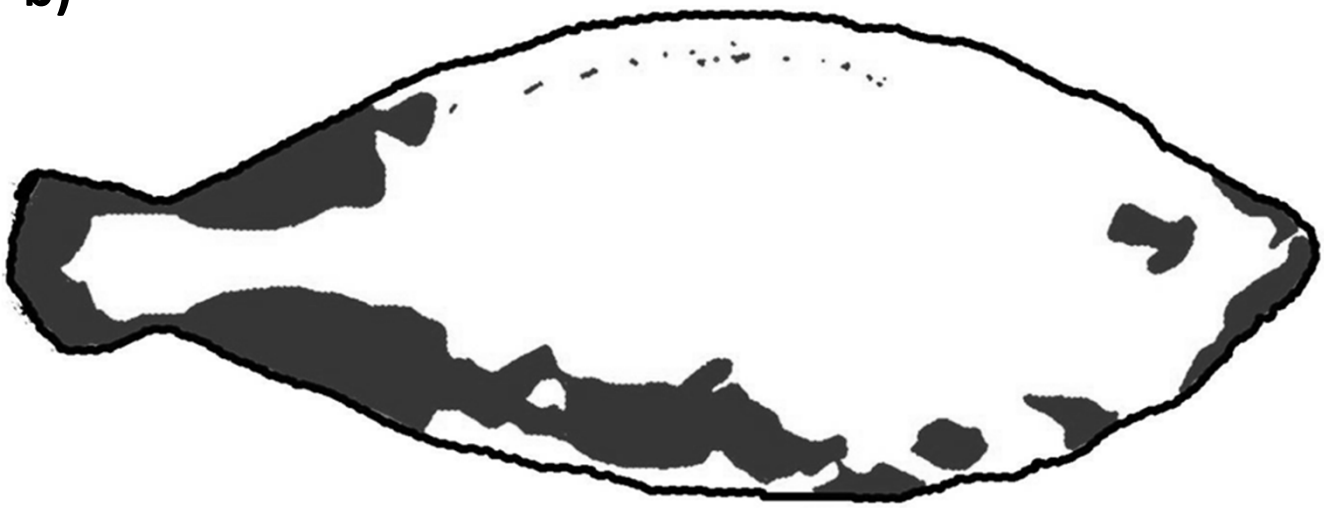


Table 1 Comparison of the initial, final body lengths (cm) and daily growth rate (mm/day) among the three groups

Group	0 week (cm)	10 week (cm)	Daily growth rate(mm/day)
Clean-started	5.5±0.2^{ab} (13)	14.9±0.3^{a} (11)	1.3±0.04^{a} (11)
Stain-started	5.3±0.1^{a} (17)	12.3±0.3^{b} (16)	1.0±0.04^{b} (16)
Pseudoalbino	5.7±0.1^{b} (10)	13.4±0.4^{b} (10)	1.1±0.06^{b} (10)
Average	5.5±0.1 (37)	13.4±0.3 (37)	1.1+0.04 (37)

*Mean + SE (*n*). Different characters indicate the presence of a statistical difference among groups in each week (P<0.05)

Table 2 Regression line and R^2 values of each week between ratio of darkening (x) and maximum ratio of darkening (y) of clean-started and stain-started fish

week	regression line(y=)	R^2
0	$4.66x+0.26$	0.210
1^{st}	$2.99x+0.20$	0.509
2^{nd}	$1.81x+0.18$	0.700
3^{rd}	$1.28x+0.19$	0.750
4^{th}	$1.17x+0.16$	0.855
5^{th}	$0.95x+0.10$	0.943
6^{th}	$0.94x+0.09$	0.965
7^{th}	$0.95x+0.06$	0.981
8^{th}	$0.97x+0.03$	0.990
9^{th}	$0.99x+0.01$	0.997